Roseovarius pacificus sp. nov., isolated from deep-sea sediment

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An aerobic, Gram-negative, ovoid to rod-shaped bacterial isolate, strain 81-2^T, was isolated from deep-sea sediment of the Western Pacific Ocean. Strain 81-2^T was motile, formed faint pink colonies, and was catalase-positive, weakly positive for oxidase and required NaCl for growth. It did not synthesize bacteriochlorophyll *a* and its DNA G+C content was 62.3 mol%. The 16S rRNA gene sequence of strain 81-2^T indicated that it was a member of the *Roseobacter* clade of the class *Alphaproteobacteria*, with moderate bootstrap support for inclusion in the genus *Roseovarius*. Its closest phylogenetic neighbour was the type strain of *Roseovarius nubinhibens*, which shared 95.8% 16S rRNA gene sequence similarity; strain 81-2^T was <95.0% similar to strains of other related species and genera. Phenotypic, chemotaxonomic and phylogenetic data support assignment of this strain to the genus *Roseovarius* as a representative of a novel species. The name *Roseovarius pacificus* sp. nov. is proposed, with strain 81-2^T (=MCCC 1A00293^T=CGMCC 1.7083^T=LMG 24575^T) as the type strain.

The *Roseobacter* clade falls within the α -3 subclass of the class *Alphaproteobacteria*, with members sharing >89% 16S rRNA gene sequence similarity. The *Roseobacter* clade is one of the major bacterial groups found in the world's oceans and members are well represented in diverse marine habitats, from coastal to open oceans and from sea-ice to sea floor (Buchan *et al.*, 2005).

The genus *Roseovarius* was created by Labrenz *et al.* (1999) and, at the time of writing, included four species isolated from various sources. *Roseovarius crassostreae* was obtained from cultured *Crassostrea virginica* (Boettcher *et al.*, 1999, 2005), *Roseovarius tolerans* was obtained from Ekho Lake (Labrenz *et al.*, 1999), *Roseovarius nubinhibens* was isolated from seawater of coastal Georgia (González *et al.*, 2003) and *Roseovarius mucosus* was isolated from a culture of the alga *Alexandrium ostenfeldii* KO287 (Biebl *et al.*, 2005). In this study, a novel strain, 81-2^T, was characterized morphologically, physiologically and biochemically, and results suggest that it should be classified as a representative of a novel species of the genus *Roseovarius*.

Strain 81-2^T was isolated from a polycyclic aromatic hydrocarbon-degrading bacterial consortium that was enriched using pyrene as sole carbon source. A sediment sample was obtained from the Western Pacific Ocean

(16° 59.9412' N 124° 58.2958' E) using a multicorer sampler at a water depth of 2682 m. The sample was enriched in a 250 ml flask containing MM medium [(l⁻¹) 3.5 g MgSO₄·7H₂O, 0.05 g CaCl₂, 24 g NaCl, 0.35 g KCl, 1.0 g NH₄NO₃, 1.0 g KH₂PO₄, 1.0 g K₂HPO₄, 0.01 g FeCl₃, 1 × 10⁻⁴ g ZnSO₄·7H₂O, 24 mg SrCl₂·6H₂O, 0.08 g KBr and 1.0 g pyrene, adjusted to pH 7.4]. After a series of transfers, a stable consortium was obtained and the culture was spread onto HLB agar plates and incubated at 28 °C for 4 days. HLB is modified Luria–Bertani medium (Sambrook *et al.*, 1989) containing increased NaCl (24 g NaCl l⁻¹). Colonies with different appearances were selected for further analysis. A colony exhibiting a pink colour on HLB was purified by transferring it to new plates and designated strain 81-2^T. Pure cultures were preserved as a glycerol suspension (20%, w/v) at -70 °C.

Bacteriochlorophyll *a* (Bchl *a*) detection was performed by spectrometric analysis of methanolic cell extracts. In the absence of light, 50 ml liquid culture was centrifuged at 5000 g for 10 min and resuspended in an ice-cold acetone/methanol solution (7:2, v/v) at -20 °C for 12 h. Cell fragments were then removed by centrifugation. The characteristic absorption peaks were observed by spectrophotometric analysis at 200–900 nm.

Extraction of genomic DNA was carried out by standard methods (Ausubel *et al.*, 1995). The DNA G+C content was determined by HPLC after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984; Mesbah & Whitman, 1989). The 16S rRNA gene was amplified by PCR using primers 16SF (positions 8–27, *Escherichia coli*

Abbreviation: Bchl *a*, bacteriochlorophyll *a*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 81-2^T is DQ120726.

A profile of the plasmid observed in strain 81-2^T (Fig. S1) is available with the online version of this paper.

Table 1. Differential characteristics of *Roseovarius pacificus* 81-2^T and type strains of related species

Strains: 1, *Rva. pacificus* sp. nov. 81-2^T (this study); 2, *Rva. tolerans* EL-172^T (Labrenz *et al.*, 1999; Biebl *et al.*, 2005); 3, *Rva. nubinihibens* ISM^T (González *et al.*, 2003; Biebl *et al.*, 2005); 4, *Rva. crassostreae* CV919-312^T (Boettcher *et al.*, 2005); 5, *Rva. mucosus* DFL-24^T (Biebl *et al.*, 2005); 6, *Leisingera methylohalidivorans* MB2^T (Schaefer *et al.*, 2002; Martínez-Cánovas *et al.*, 2004; Martens *et al.*, 2006); 7, *Phaeobacter gallaeciensis* BS107^T (Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006); 8, *Marinovum algicola* ATCC 51440^T (Lafay *et al.*, 1995; Labrenz *et al.*, 1999; Martínez-Cánovas *et al.*, 2004; Martens *et al.*, 2006); 9, *Silicibacter pomeroyi* DSS-3^T (González *et al.*, 2003); 10, *Sagittula stellata* E-37^T (González *et al.*, 1997; Labrenz *et al.*, 1999); 11, *Antarctobacter heliothermus* EL-219^T (Labrenz *et al.*, 1998, 1999); 12, *Roseobacter denitrificans* DSM 7001^T (Shiba, 1991; Labrenz *et al.*, 1999; Martens *et al.*, 2006); 13, *Sulfitobacter mediterraneus* DSM 12244^T (Pukall *et al.*, 1999; Martínez-Cánovas *et al.*, 2004). +, Positive; −, negative; v, variable; w, weak; ND, not determined; TR, trace.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Cell shape	Ovoid to rods	Short rods	Short rods	Ovoid to rods	Short rods	Rods/ ovoid rods	Ovoid rods	Ovoid rods	Rods	Rod	Rod	Ovoid rods	Rods
Source of isolation	Western Pacific Ocean	Antarctic lake	Seawater (Caribbean Sea)	Animal (<i>Crassostrea virginica</i>)	Marine dinoflagellate	Seawater	Seawater	Marine dinoflagellate	Seawater	Seawater	Ekho Lake	Seaweed	Seawater
Colony colour	Faintly pink	Red	Cream margin and brown centre	Pink-beige	Whitish to faintly pink	−	Brown	Beige-pink	Cream	Cream	Brown-yellow	Pink	Cream
Motility	+	+	+	+	−	+	+	+	+	−	v	+	+
Flagella	Subpolar	Not found	ND	Subpolar to lateral	Not found	Subpolar	Polar	Subpolar	Polar	+	Subpolar	Subpolar	Subpolar
Oxidase	+	w	v	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	−	−	−	−	−	−	−	−	−	−	+	−	−
Bchl <i>a</i>	−	+	Not found	−	TR	−	−	−	−	−	−	+	−
Na ⁺ requirement	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Growth in NaCl (%):													
Range	1–15	1–15	0.8–4.7	ND	0.3–10	1–6	0.6–11.7	0.5–12	ND	ND	1–10	ND	0.2–8.0
Optimum	2–12	1–8	1.1–2.3	1–1.5	1–7	3	1.2	ND	0.6–2.3	ND	2–6	ND	1.5–2
Optimum growth temperature (°C)	25	8.5–33.5	30	34	31	27	23–27	25–30	30	30	16–26	20–30	17–28
Growth at:													
4 °C	w	+	−	−	−	+	−	−	−	−	ND	+	+
37 °C	+	+	+	+	+	−	+	+	+	+	+	−	−
pH optimum	6.2–8.5	6.2–9.0	ND	6.5–8.0	6.5–8.8	7.7	7.0	7.5	ND	7.5	6.9–7.8	7.0–8.0	7.0–7.5
Aesculinase	−	ND	ND	−	ND	ND	ND	+	ND	ND	ND	ND	ND
β-Galactosidase	−	ND	ND	−	ND	ND	ND	+	ND	ND	ND	ND	ND
Gelatinase	−	−	−	−	+	−	−	+	+	−	+	+	+
Amylase	−	−	−	ND	−	−	−	+	−	−	v	−	ND
Tween 80	−	−	−	ND	−	−	−	−	−	−	−	+	ND
Reduction of:													
Nitrate to nitrite	−	−	−	+	ND	−	−	−	−	−	+	+	−

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Nitrite to N ₂	—	—	—	+	ND	—	—	—	—	—	+	—	—
Indole production	—	—	ND	ND	—	ND	ND	—	ND	—	—	+	ND
Acid production from glucose	—	—	—	—	—	—	ND	—	—	—	—	ND	—
C source oxidized and/or utilized:													
Citrate	W	—	—	—	—	—	—	+	+	+	+	+	+
Glucose	—	—	+	—	—	—	+	+	+	+	+	+	+
Fructose	—	ND	—	—	—	—	+	+	—	+	+	+	ND
Ethanol	+	ND	+	ND	—	ND	ND	ND	+	ND	+	ND	ND
Glycerol	+	ND	—	+	+	—	+	—	+	+	—	—	+
Major fatty acid	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c	ND	C _{18:1}	C _{18:1} ω7c	C _{18:1} ω7c
Susceptible to:													
Penicillin G	+	+	ND	ND	+	+	—	+	ND	+	+	ND	ND
Tetracycline	+	+	ND	ND	—	ND	ND	W	ND	+	+	ND	ND
DNA G+C content (mol%)	62.3	63.3–63.4	66	59	62.9	60.5	57.6–58.0	64–65	68.0±0.1	65	62.3–62.8	59.6	59

numbering; 5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (positions 1512–1493; 5'-ACGGCTACCTTGTT-ACGACT-3'). Thermal cycles were performed on a T3 thermal cycler (Biometra). Amplified DNA fragments were purified and recovered by using an EZNA Cycle-Pure kit (Omega Bio-Tek). Sequencing was carried out on a model 3730 automated DNA sequencer using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

The sequences of strain 81-2^T and reference bacterial species that were obtained from GenBank were aligned manually with DNAMAN software, version 6 (Lynnon Biosoft). Phylogenetic analysis of 16S rRNA gene sequences was also performed with DNAMAN software.

The morphological, physiological and biochemical characteristics of strain 81-2^T are given in the species description (see below) and Table 1. Properties that differentiate strain 81-2^T from related members of the *Roseobacter* clade are shown in Table 1. Strain 81-2^T grew well on HLB medium. Colonies were whitish to faintly pink, circular, moist-appearing, convex, and had entire margins and smooth surfaces. When grown in a medium containing 10 g peptone l⁻¹ and 5 g yeast extract l⁻¹, the strain grew readily in 2–12 % NaCl, although growth was slower at 2 and 12 % NaCl. No growth occurred in the absence of NaCl. For carbon source utilization tests (described below), a salinity of 2.4 % NaCl was used. Within the temperature range tested (4–45 °C), growth was observed throughout, with maximum growth observed at 25 °C. Growth was equally good at an initial pH between 6.2 and 8.5, but not at pH 5.2 or 9.1. Strain 81-2^T did not produce Bchl *a* in the dark as the characteristic absorption peak between 400 and 900 nm was not detected. Agarose gel electrophoresis of chromosomal DNA showed that strain 81-2^T contained a small plasmid that has not been reported in other species of the genus (see Supplementary Fig. S1 in IJSEM Online). The plasmid could be digested by *Hind*III (Takara), giving a linear product of ~5.0 kb.

A comparison of 16S rRNA gene sequences revealed highest similarities between strain 81-2^T and type strains of the following species: *Rva. nubinhbens* (95.8 %); *Rva. crassostreae* (94.4 %); *Rva. tolerans* (94.9 %); *Rva. mucosus* (94.3 %); *Phaeobacter gallaeciensis* (94.3 %); *Roseobacter litoralis* (92.7 %); and *Marinovum algicola* (94.4 %). The strain joined the clade comprising species of the genus *Roseovarius*. Following these discoveries, detailed phylogenetic analysis of strain 81-2^T and strains of species of the *Roseobacter* clade with validly published names was performed; the phylogenetic tree based on analysis of the 16S rRNA gene (1428 bp) indicates that strain 81-2^T, together with other *Roseovarius* species, formed a clearly separate group within the *Roseobacter* clade (Fig. 1). The same topology was also found in trees based on maximum-likelihood and maximum-parsimony algorithms.

The oxidation of various substrates was determined by using the Biolog system as described previously (Ivanova *et al.*, 1998). GN2 Microplates were selected and, after

Fig. 1. A complete neighbour-joining tree showing the phylogenetic position of strain 81-2^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.05 substitutions per nucleotide position.

adding 150 µl liquid culture with an NaCl concentration of 2.4 %, plates were incubated at 25 °C. Tests for utilization of various other organic substrates as sole carbon sources at a concentration of 0.1 % (w/v) were performed in 5 ml MM medium. Catalase and oxidase activities and the hydrolysis of starch were determined as described by Cowan & Steel (1965). Aesculin hydrolysis and nitrate reduction were investigated as described by Lányi (1987). Other physiological and biochemical tests were performed with the API 20E system (bioMérieux) using API medium with an NaCl concentration of 24 g l⁻¹.

Susceptibility to different antibiotics was tested on HLB plates by using discs containing the following antibiotics (µg): fortum, 30; cefuroxime, 30; cephadrin, 100; cefazolin, 30; cefalexin, 30; piperacillin, 100; carbenicillin, 100; ampicillin, 10; oxacillin, 1; penicillin, 10; erythromycin, 15; minomycin, 30; vibramycin, 30; tetracycline, 30; neomycin, 30; kanamycin, 30; gentamicin, 10; amikacin, 30; cefobid, 75; rocephin, 30; vancomycin, 30; polymyxin B, 300; ofloxacin, 5; midecamycin, 30; ciprofloxacin, 5; norfloxacin, 10; furazolidone, 300; co-trimoxazole, 75; chloromycetin, 30; and clindamycin, 2.

Cell shape and motility were observed by light microscopy (Olympus). Cell size and more detailed morphology were

determined by transmission electron microscopy, for which cells were suspended and absorbed on a Formvar, carbon-coated grid, then stained with phosphotungstic acid; after air-drying, the grid was examined by using an H-600 transmission electron microscope (Hitachi). Cells were motile and ovoid to rod-shaped, 0.48–0.58 × 0.94–1.74 µm in size. In addition, cells possessed at least one subpolar flagellum (Fig. 2).

Cellular fatty acid analysis was carried out as described by Mrožik *et al.* (2004). Analysis of fatty acid methyl esters

Fig. 2. Transmission electron micrographs of negatively stained cells grown on HLB agar for 72 h. Bars, 1 µm.

was performed on a GC-MS (QP2010; Shimadzu) equipped with an RTX-5MS column. Peaks were identified with pre-installed software, GC-MS Postrun analysis software (version 2.10; Shimadzu). The fatty acid profile of strain 81-2^T comprised unsaturated, straight-chain and hydroxy fatty acids. This fatty acid profile was similar to those of other *Roseovarius* species, although there were differences in the proportions of some fatty acids, perhaps because of differences in cultivation conditions (González *et al.*, 2003; Table 2). As in most members of the class *Alphaproteobacteria*, C_{18:1}ω7c was the predominant component, amounting to more than 70 % total fatty acid content. C_{12:0} 3-OH was present in both strain 81-2^T and *Rva. nubinihibens*, whereas *Rva. mucosus* and *Rva. tolerans* contained C_{12:1} 3-OH.

Distance analyses consistently placed strain 81-2^T in the genus *Roseovarius* as shown in Fig. 1. However, a reliability value of 59 % indicated that the branching pattern was not very stable and did not provide strong support for inclusion of this organism in the genus *Roseovarius*. A comparison of phenotypic characteristics showed that properties of strain 81-2^T were consistent with those of members of the genus *Roseovarius* (Table 1) and its DNA G + C content was in the range 62–64 mol% (Labrenz *et al.*, 1999). Thus, despite the low bootstrap value to the *Roseovarius* group, the above analyses support the proposal that strain 81-2^T represents a novel species of the genus *Roseovarius*.

In conclusion, phenotypic and phylogenetic analyses support placement of this strain as a novel species in the

genus *Roseovarius*. The proposed name is *Roseovarius pacificus* sp. nov.

Description of *Roseovarius pacificus* sp. nov.

Roseovarius pacificus (pa.ci'fi.cus. M.L. adj. *pacificus* pertaining to the Pacific Ocean from which the type strain was isolated).

Cells are aerobic, non-spore-forming, Gram-negative and ovoid to rod-shaped, 0.48–0.58 × 0.94–1.74 μm. Actively motile; at least one subpolar flagellum may be present. On HLB agar plates, small, wettish, smooth, faint pink colonies develop in 3–5 days. Optimal growth is observed at 25 °C, salinities of 2–12 % and pH between 6.2 and 8.5. There is an absolute requirement for sodium ions. Does not contain Bchl *a*. Cells are positive for oxidase and catalase, and negative for nitrate reduction, hydrolysis of starch and gelatin liquefaction. H₂S and indole are not produced. In assays with the API 20E system, lysine decarboxylase is present, but β-galactosidase, arginine dihydrolase, ornithine decarboxylase, urease and tryptophan deaminase are absent. Voges–Proskauer test is positive. Oxidizes the following substrates: methyl pyruvate, monomethyl succinate, acetic acid, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, succinamic acid, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, L-ornithine, L-proline, L-pyrogutamic acid, L-serine, γ-aminobutyric acid, 2-aminoethanol, ethanol and glycerol. The following carbon sources are oxidized weakly: Tween 40, *cis*-aconitic acid, citric acid, *p*-hydroxyphenylacetic acid, D-saccharic acid, bromosuccinic acid, D-alanine, DL-carnitine and urocanic acid. Susceptible to fortum, cefuroxime, cephradine, cefazolin, cefalexin, piperacillin, carbenicillin, ampicillin, penicillin G, minomycin, vibramycin, tetracycline, neomycin, kanamycin, gentamicin, amikacin, cefobid, rocephin, polymyxin B, ofloxacin, midecamycin, ciprofloxacin, norfloxacin and chloromycetin, but not to oxacillin, erythromycin, vancomycin, furazolidone, co-trimoxazole or clindamycin. The major fatty acid (>70 % total fatty acids) is C_{18:1}ω7c. Other phenotypic characteristics are given in Table 1.

The type strain is 81-2^T (=MCCC 1A00293^T=CGMCC 1.7083^T=LMG 24575^T), isolated from deep-sea sediment of the Western Pacific Ocean. The DNA G + C content of the type strain is 62.3 mol% (determined by HPLC).

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Table 2. Fatty acid composition (%) of some species of the genus *Roseovarius*

Strains: 1, *Rva. pacificus* sp. nov. 81-2^T (data from this study); 2, *Rva. tolerans* EL-172^T (Labrenz *et al.*, 1999); 3, *Rva. nubinihibens* ISM^T (González *et al.*, 2003); 4, *Rva. crassostreae* (12 strains; Boettcher *et al.*, 2005); 5, *Rva. mucosus* DFL-24^T (Biebl *et al.*, 2005). –, Not detected.

Fatty acid	1	2	3	4	5
C _{10:0} 3-OH	—	—	—	2.1 ± 0.3	0.7
C _{12:0}	4.24	—	3.3	—	3.1
C _{12:0} 2-OH	—	2.4	—	—	2.5
C _{12:0} 3-OH	4.56	—	1.5	—	—
C _{12:1} 3-OH	—	3.6	—	—	1.7
C _{16:1} ω7c	—	—	1.0	—	—
C _{16:1} ω9c	1.35	0.8	—	—	0.8
C _{16:0}	6.21	6.2	9.8	3.6 ± 0.9	13.1
C _{17:0}	—	—	—	—	—
C _{18:0}	3.8	0.8	0.6	0.7 ± 0.3	1.8
Methyl C _{18:0}	—	—	—	—	2.1
C _{18:1} ω7c	73.95	70.2	76.4	85.2 ± 1.7	69.4
Methyl C _{18:1} ω7	—	—	—	0.8 ± 0.2	2.1
Cyclo C _{19:1}	—	—	5.3	—	3.7
C _{18:2}	—	10.6	—	—	—

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